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Early Complete Hydatidiform Moles Contain Inner Cell Mass Derivatives

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In four cases of early complete hydatidiform moles, confirmed to be androgenetic in origin by DNA studies, we have identified nonchorionic inner cell mass derived structures which are not commonly observed in specimens of later gestational age. These structures include nucleated red blood cells, endothelial cells, stromal macrophages, amnion and yolk sac. The latter four structures were confirmed by specific immunocytochemical stains. Recognition that such structures can accompany complete hydatidiform moles has both theoretical and practical significance. From a theoretical perspective, it demonstrates that the maternal genome is not required for the initiation of amniogenesis, development of the yolk sac, vasculogenesis, or hematopoiesis. From a practical perspective it emphasizes that complete hydatidiform moles, with their markedly increased risk of subsequent choriocarcinoma, cannot be excluded based on the finding of "fetal structures." Am. J. Med. Genet. 70:273–277, 1997. © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Complete hydatidiform moles (CHM) are aberrant gestations which lack fetal tissues, display hydropic degeneration of chorionic villi and manifest diffuse exuberant proliferation of the placental trophoblastic cells. The trophoblastic proliferation in CHM markedly increases the risk for subsequent trophoblastic malignancies such as choriocarcinoma and placental site trophoblastic tumor. CHM generally have a diploid karyotype (46,XX or 46,XY) that is entirely paternal in origin [Kajii and Ohama, 1977]. Thus, CHM are believed to represent an example of the phenotypic consequences of genomic imprinting in humans. Furthermore, they provide evidence that the paternal genome plays a critical role in placental development.

The existence of nonchorionic tissues in complete hydatidiform moles is not widely appreciated and has never been unequivocally documented in genotypically proven cases [Szulman and Surti, 1978; Vassilakos et al., 1977]. The absence of such structures has led to speculation as to whether they ever develop in the absence of the maternal genome. This suggests that a uniparental paternal genome may primarily support trophoblast development and that structures derived from the inner cell mass including the embryo proper as well as nonchorionic adnexal and hemangioblastic tissues require a biparental genome.

In this study, we describe five cases of early complete hydatidiform moles which have been confirmed by DNA analysis to be androgenetic in origin, and in four of the cases provide evidence that such samples may contain inner cell mass structures including amnion, yolk sac, fetal endothelial cells, macrophages, and nucleated red blood cells.

MATERIALS AND METHODS

Case selection: The index case was retrospectively identified when the patient had a repeat curettage for persistently elevated HCG titers 45 days following evacuation of a presumed missed abortion. The initial specimen had not been diagnosed as a molar pregnancy. The repeat curettings, however, showed classical features of complete mole (villous cisterns and circumferential trophoblastic hyperplasia) and, upon genotypic analysis using microsatellite polymorphisms, was androgenetic in origin (data not shown). This prompted re-evaluation of the initial curettage sample (index case, Table I). Genotypic analysis indicated that it was also androgenetic in origin but, surprisingly, it was genetically distinct from the later molar sample, indicating that the two samples were derived from separate fertilization events. Recurrent complete moles are a recognized clinical entity [Roberts et al., 1994].

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TABLE I. Summary of Results for the Microsatellite Analysis

Case	Tissue[a]	Microsatellite alleles									Origin
		SPN	D21S11	IL2RB	D2S172	D14S49	D7S550	D6S305	D10S185	STS	
Control 1 (SAB)	F	AB	ABC	AB	—	—	—	—	—	—	Biparental
	M	BC	BC	BC	—	—	—	—	—	—	
Control 2 (EAB)	F	AB	AB	AB	—	—	—	—	—	—	Biparental
	M	BC	BC	BC	—	—	—	—	—	—	
Index case[b]	F	AA[d]	AA	—	AA	AA	AA	AA	AA	AA	Androgenetic
	M	BC	AC	AB	BC	BC	BC	BC	BC	AA	
Case 2[b]	F	AA	AA	AA	AA	AA	AA	AA	—	AA	Androgenetic
	M	BC	BC	AB	AB	BC	BB	BB	—	AA	
Case 3[b]	F	AA	AA	AA	—	AA	AA	AA	AA	AA	Androgenetic
	M	BC	AA	BC	—	BB	BC	BC	BC	AA	
Case 4[b]	F	AA	AA	AA	AA	AA	AA	AA	—	AA	Androgenetic
	M	BC	BC	AB	AA	BB	AB	AB	—	AA	
Case 5[c]	F	AA	AA	—	AA	AA	AA	AA	AA	AA	Androgenetic
	M	BC	BC	—	BB	BC	AB	BC	BC	AA	

[a]F, fetal; M, maternal.
[b]Ascertained at University Hospitals, Cleveland.
[c]Ascertained at Brigham and Women's Hospital, Boston.
[d]In the absence of cytogenetic studies, we assume that the mole is diploid; however, we cannot formally exclude haploidy or polyploidy.

Histological analysis of the initial curettage of the index case revealed features which differed from those typically used to diagnose later complete moles. These criteria were then used to identify three additional cases (cases 2, 3 and 4, Table I) from specimens submitted to the Department of Pathology at University Hospitals of Cleveland over the past three years. Genotypic analysis confirmed that all cases were androgenetic in origin. Histological criteria for diagnosis of early complete moles, which are described elsewhere [Keep et al., 1996], were developed using these cases. Three of the four cases showed nonchorionic structures which are the focus of this report.

One additional case (case 5, Table I) which showed nucleated red blood cells, was ascertained in a review of molar pregnancies diagnosed at Brigham and Women's Hospital, Boston and was confirmed by microsatellite analysis to be androgenetic in origin, consistent with the diagnosis of a complete molar gestation.

The five cases ranged in gestational age from 6.5 to 11 weeks based on last menstrual period. All were diagnosed clinically as missed abortions and curetted on that basis.

DNA studies: All cases were analyzed for parental chromosome contribution by PCR amplification of microsatellite markers, using DNA extracted from maternal and fetal tissue dissected from paraffin blocks. Tissue samples from two controls, a spontaneous abortion (SAB) and an elective abortion (EAB), also were obtained and analyzed. For all samples, the tissues were dewaxed in xylene, rehydrated in an ethanol series and incubated in a lysis solution containing proteinase K. DNA then was extracted with phenol:chloroform and precipitated with ethanol [Lane et al., 1993].

For each case, DNA was amplified using primers for eight microsatellite DNA polymorphisms (Table I), with the loci chosen for being highly polymorphic. For each marker, 150-500 ng of DNA was amplified in a total volume of 25 ul, using standard conditions and "low touchdown" (for D21S11, D2S172, D7S550, and D14S49) or "high touchdown" (for SPN, IL2RB, D6S305 and D10S185) protocols [Don et al., 1991]. PCR products were denatured at 95°C for 5 minutes and separated by polyacrylamide gel electrophoresis. To determine the sex chromosome constitution, a ninth primer set, which amplifies both the X-linked STS locus and the Y-linked STS pseudogene but yields different sized products for the two loci, was also used. For these loci, nested-PCR was performed as described by Zhang et al. [1992].

Androgenetic (paternal) origin was indicated by the exclusion of maternal alleles in the fetal tissue and in each case, we required that at least three markers be informative before reaching a conclusion on the paternal origin of the fetal genome.

Immunohistochemistry: Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using monoclonal antibodies to CD68 (Dako), HAM56 (Enzo), and CAM 5.2 (Becton-Dickinson), polyclonal antibodies to factor VIII (Dako) and alpha fetoprotein (Dako), and Ulex europeus lectin (Dako). Briefly, the specimens were sectioned at 3-4 um on Silane-coated slides. Sections were deparaffinized in xylene and rehydrated in descending grades (95%-50%) of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Pretreatment by microwave antigen retrieval in 10 mM citrate was performed for Factor VIII and Ulex europeus. CD68 and CAM 5.2 were treated with pepsin and trypsin respectively. No pretreatment was necessary for HAM56 or alpha fetoprotein. All sections were incubated in 10% normal goat serum for 10 minutes at room temperature to prevent nonspecific binding. Immunostaining was performed using the streptavidin-biotin-peroxidase method. Primary antibodies were incubated at predetermined dilutions for one hour at 37°C. Biotinylated secondary antibody and streptavidin-peroxidase steps were performed using an antibody

detection kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were counterstained with Harris' Hematoxylin.

RESULTS

DNA studies: The results of the DNA analysis are summarized in Table I. For the two control samples, we were able to demonstrate the presence of both paternal and maternal alleles thus validating the reliability of the tissue procurement technique. For each of the five molar cases, we identified only paternally-derived alleles; thus each case was androgenetic in origin. Further, each case appeared to be totally homozygous.

Immunohistochemistry: The results are summarized in Table II. In the index case and case 2, subpopulations of chorionic villi showed stromal capillaries lined by Factor VIII-positive, Ulex Europeus-positive endothelial cells (Fig. 1a). Furthermore, in one case (case 5) nucleated red blood cells were observed in the endothelial-lined spaces (Fig. 1b). In the index case and case 4, intermixed with fibroblasts in the villous stroma were clusters of HAM56-positive, CD68-positive villous macrophages (Hofbauer cells) (Fig. 1c). In addition, two cases (cases 4 and 5) contained placental adnexal structures not generally observed in later complete moles. Cytokeratin-positive amnion (CAM 5.2) was found adherent to the surface of the chorionic plate in two cases (Fig. 1d) and, in one case, a well formed yolk sac with a central core of alpha fetoprotein-positive endodermal epithelial cells was observed (Fig. 1e). In only one case (case 3), there was no evidence of nonchorionic tissues.

DISCUSSION

In this report, we describe early CHM that have been confirmed to be androgenetic in origin but contain inner cell mass derivatives not commonly observed in molar specimens of later gestational age. The origin of these moles, however, appears to be similar to the origin of classic complete moles which are thought to arise by the fertilization of an anucleate egg by a single haploid sperm followed by endoreduplication to restore diploidy [Jacobs et al., 1980]. Since there was no evidence of heterozygosity for at least eight loci for each case, it seems likely that each arose from the fertilization of an anucleate egg by a normal haploid sperm.

The presence or absence of such tissues as nonchorionic adnexal elements and hemangioblastic tissues in CHM has been controversial and never adequately documented. Earlier studies on the morphology of complete moles described placental chorionic villi with trophoblastic hyperplasia; however, tissues embryologically derived from the inner cell mass, including the embryo proper, fetal red blood cells and amnion were not observed [Szulman and Surti, 1978].

Due to the increasing use of ultrasound in the diagnosis of moles, complete moles are now being evacuated much earlier and it has been suggested that these earlier specimens contain histological features different from classical moles. For example, chorionic vessels that disappear with the development of hydrops were described in such early moles [Paradinas, 1994], and in a larger study of 149 complete moles by the same authors, only 12% of complete moles were avascular [Paradinas et al., 1996]. Furthermore, amnion was present in 17% of specimens and nucleated red cells were found in 9% of specimens. However, these studies did not include molecular analysis of the parental origin of the chromosome complement. Our immunocytochemical studies of early complete moles confirmed to be androgenetic in origin on the basis of DNA marker studies, have helped to clarify these issues.

First, we identified typical amnionic epithelium (CAM 5.2-positive) in close contact with amnionic connective tissue and the underlying chorion. Although extraembryonic, amnion develops later and has an embryologic origin that is distinct from chorion and trophoblast. The amnion originates from the inner cell mass with its mesodermal layer from the hypoblast and its ectodermal layer from the epiblast [Luckett, 1978].

Second, we identified a typical yolk sac consisting of mesodermal cells ensheathing a central core of alpha fetoprotein-positive endodermal epithelial cells in one specimen. The presence of a yolk sac, the earliest site of embryonic hematopoiesis, helps to explain our finding of villous stromal macrophages (CD68- and HAM 56-positive) and circulating fetal nucleated red blood cells in early CHM. Yolk sac and hematopoietic elements are both derived from the hypoblast of the inner cell mass [Bianchi et al., 1993].

Finally, we found endothelial-lined capillaries (Factor VIII- and Ulex Europeus-positive). There has been considerable controversy over the years regarding the origin of villous capillaries. The original proposal that they delaminate from trophoblast would be consistent with their presence in early complete moles [Hertig, 1968], but is generally not believed to be true at this time [Benirschke and Kaufmann, 1995]. A later theory that they are derived from fetal allantoic mesoderm is difficult to reconcile with the absence of embryonic tis-

TABLE II. Summary of the Results of the Immunohistochemical Analysis*

	Tissue-antibody				
Case	Amnion-CAM 5.2	Yolk sac-AFP	Endothelium-U. europeus/ factor VIII	Macrophages-HAM 56/CD68	Nucleated red blood cells
Index case	–	–	+	+	–
Case 2	–	–	+	–	–
Case 3	–	–	–	–	–
Case 4	+	+	–	+	–
Case 5	+	–	NT	NT	+

*+, present; –, absent; NT, not tested.

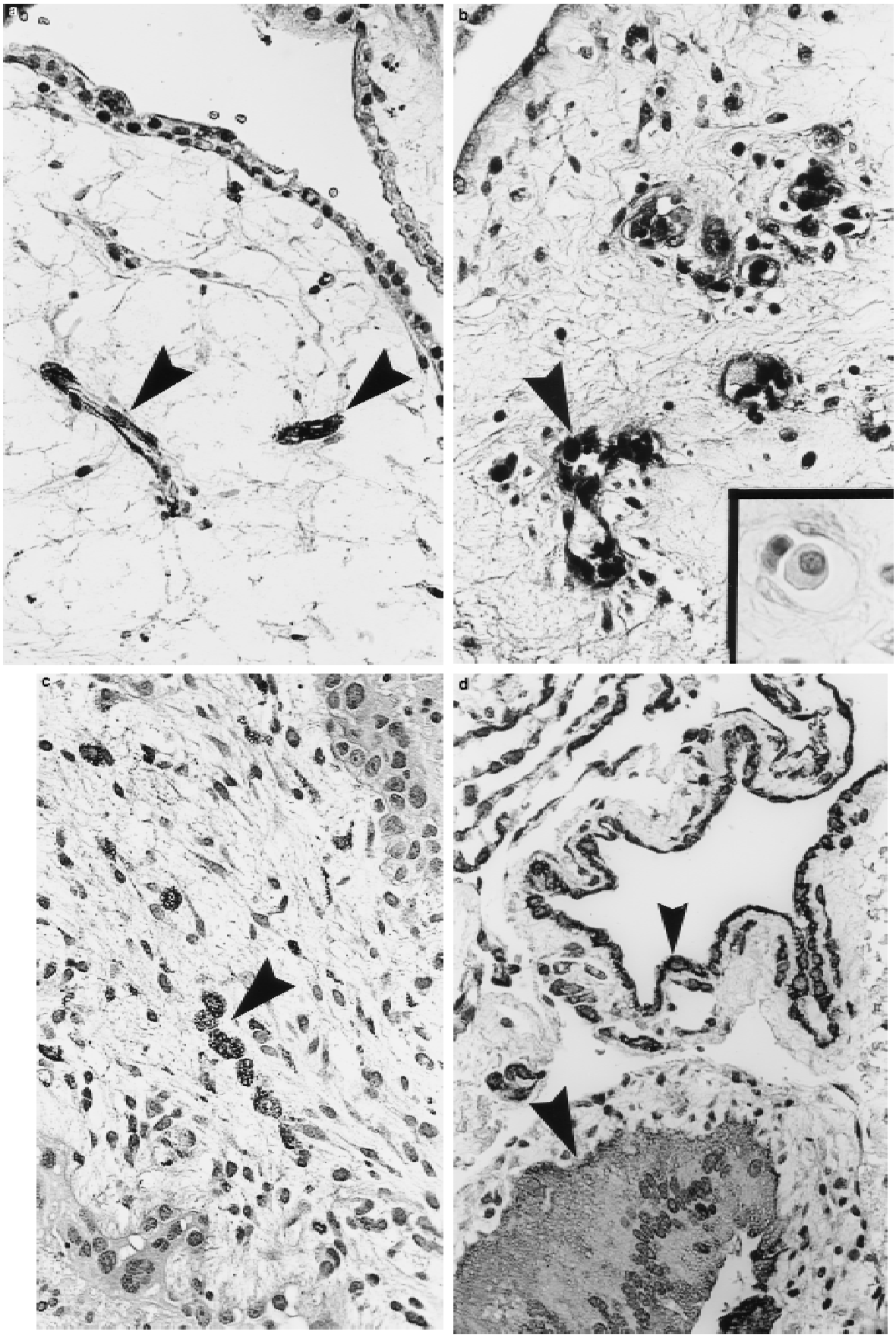

Fig. 1.

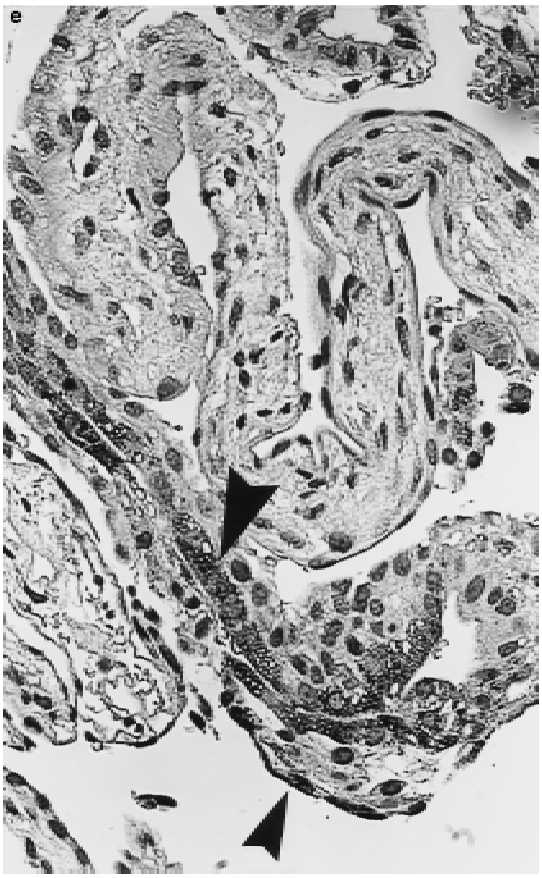

Fig. 1. **a:** Factor VIII immunostaining of endothelial cells lining villous stromal capillaries in ECM (arrows). Original magnification ×2,240. **b:** Nucleated blood cells in fetal capillaries between amnion and chorion in ECM (arrow). Original magnification ×3,400. **Inset** shows the typical nucleated red blood cell morphology consisting of a round central hyperchromatic nucleus surrounded by glassy intensely eosinophilic cytoplasm and a distinct cell border. Original magnification ×4,600. **c:** CD68-positive vacuolated macrophages clustered within the cellular stroma of ECM (arrow). Original magnification ×1,400. **d:** CAM 5.2 positive squamoid amnionic epithelium in ECM (small arrow). Note the underlying loose fibroblasts separated from underlying fibrous chorion by a typical clear space. Also note proliferative CAM 5.2 positive syncytiotrophoblast on the underside of the chorion (large arrow). Original magnification ×1,400. **e:** Alpha fetoprotein positive yolk sac in ECM consisting of a bilayer of positively staining cuboidal epithelial cells (large arrow), scant surrounding mesenchyme, and an outer layer of amnion (small arrow) (CAM 5.2 positive, not shown). Original magnification ×1,400.

sue in our cases [Luckett, 1978]. Most recently it has been proposed that villous vessels are derived from the loose mesenchyme surrounding the endodermally derived yolk sac [Bianchi et al., 1993]. This histogenesis would be consistent with our finding of a yolk sac in one case (Table II).

Our observations on early CHM demonstrate that, although the majority of tissues derive from extraembryonic ectoderm, other embryonic germ layers also contribute to the early androgenetic human conceptus. Failure to find inner cell mass derived structures in later CHM may relate either to lack of maintenance factors or active programmed cell death. One possibility is that maintenance or antiapoptic factors could be the products of paternally imprinted genes (maternally expressed loci) which would not be expressed in androgenetic molar conceptuses. In any case, our observations closely parallel studies of murine uniparental zygotes produced by in vitro nuclear transfer [Surani et al., 1984; McGrath and Solter, 1984]. These murine androgenetic conceptuses show proliferation of trophoblast but also stunted embryonic tissue, while gynogenetic conceptuses have poor growth of trophoblast but greater development of the embryo proper.

A practical conclusion deriving from this study is that the finding of capillaries, nucleated red blood cells, amnion, or yolk sac does not exclude the diagnosis of CHM. Failure to recognize early CHM can have significant impact for the patient's health as demonstrated by the index case who presented later with recurrent gestational trophoblastic disease and also a subsequent case of early complete mole, not included in this report, which was followed two months later by a hysterectomy showing choriocarcinoma.

Finally, an additional theoretical concern relates to the significance of finding fetal red cells in moles since in current clinical practice Rhogam is not given to Rh negative women with complete moles. We should emphasize that in contrast to other reports [Paradinas et al., 1996] fetal red cells were rarely seen in our material and when present were few in number.

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